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(54) **Method for the manufacture of proteaseinhibitors from potato-fruitwater.**

(57) A method for the manufacture of proteaseinhibitors from potato-fruitwater wherein potato juice from the potato starch production is used as raw material for the manufacture. Potato juice is otherwise a waste which gives environmental problems in the production of potato starch.

The method is gentle such that the inhibitor proteins are not damaged. The method is also comprising further purification and concentration of the proteaseinhibitors.

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This invention concerns a method for extracting and concentrating proteaseinhibitors from potato-fruitwater which is a waste from the production of potatostarch.

Poteases are enzymes which hydrolyse proteins into aminoacids, and proteaseinhibitors are compounds which inhibit this reaction. Proteaseinhibitors preserve raw materials of animal origin and materials which contain or are contaminated by proteases, by inhibiting the hydrolysis of proteins to aminoacids. In this way the proteins are made unavailable to the digestionssystem of the microorganisms.

Owing to a high content of proteases, prey-containing fish gives reduction in quality as well as difficulties in processing when used as raw material for the fish-meal and fish-oil industry.

In laboratory experiments and in wholesale tests under actual fishing, it has been found that proteaseinhibitors extracted from potatojuice efficiently hinder the dissolving of proteins and the formation of biogenic amines in prey-containing fish for industrial use. Potato-inhibitors from the juice of the potato make prey-containing fish keep better in storage. For the Fishmealindustry this means that problematic rawmaterial of low storageability may be upgraded to production of fishmeal of a better quality than meal produced from fish without this proteaseinhibitor treatment.

Published information is available that the potato contains inhibitors for chymotrypsin, carboxypeptidase A and B and trypsin. By analysing for these proteases we have followed the movement and yield of these inhibitors through different steps of the process which we are going to describe.

The juice of the potato tubers is a waste which gives environmental problems in the production of potato starch. In the professional vocabulary the undiluted juice from the potato tuber is called potato fruitjuice, whereas the diluted juice is designated potato fruitwater. Both have a high content of organic materials which give rise to high oxygen demand (high BOD and COD) in waste water from the potato starch plants. The potato fruit water also contains phosphorous- and nitrogen-compounds which fertilize the recipients.

Norwegian potato starch factories and gradually many potato starch factories in other countries use methods whereby the potato fruitwater is extracted with little or practically no dilution. The procedure for extracting and concentrating proteaseinhibitors, for which procedure protection by patent is applied for, presupposes the use of little diluted fruitjuice as raw material.

Concentrating potato fruitwater to be used as feed supplement by evaporation or reverse osmosis is used by some potato starch manufacturers. Reverse osmosis is not as energy demanding as evaporation, does however demand that the potato

fruitwater is pretreated and filtered clear to avoid clogging of the membranes which hold inorganic salts and low molecular organic components back in the concentrate.

Even if the proteaseinhibitor concentrate is primarily intended to be used for the preservation of fish, it may also be used to inhibit unwanted protease activities in other raw materials of animal origin and products with such unwanted activities.

It will also be described a method for further purification and concentration of proteaseinhibitors made from potato fruitjuice.

In the medical litterature it is claimed that some proteaseinhibitors may cure or help cure certain types of illness. The purified and concentrated proteaseinhibitors may be the basis for further fractionation and purification to investigate the possibilities for this type of use.

According to this invention, the raw material for the extraction of proteaseinhibitors is the fruit juice from the production of potato starch. Small scale extraction of proteaseinhibitors from potatoes is described in the litterature. For extraction of potatojuice and production of proteaseinhibitors on a laboratory scale, whole potato tubers have allways been used, and the production of the juice has been done under controlled ideal conditions. It does however turn out that the proteaseinhibitors in question only to a slight degree are destroyed during the industrial starch production when the starch is washed out from the finely ground potatoes with sulfurdioxide containing potatofruitjuice, and that the wastewater after the extraction of the starchgranules is an excellent source for the production of proteaseinhibitors.

The dry matter content of the potatoes varies depending on the potato variety and growthconditions between 20 and 30 g dry matter/100 g potato tuber. The dry matter of the potato tubers contains considerable quantities of protein, 5-6 g protein/100 g dry matter. The potato protein is composed of a complex group of proteins and polypeptides with strong inhibitor activities towards the most important pancreas-proteases of higher animals.

As a mean, the potato tuber contains 3/4 water and 1/4 dry matter. The starch and the fibers occur as particulate material, and make up the larger part of the dry matter. The liquid phase has varying content of dissolved dry matter, and the proteaseinhibitors make up part of this dissolved dry matter. The starch is available as granules suspended in the potatojuice which again is enclosed in cells. The extraction of the starch starts by grinding the potato tubers in such a way that the cell walls are broken and the juice with starch granules and fibers is made free and make a slurry. The starch granules and the fibers are afterwards mechanically separated from the potato fruitjuice. The juice contains phenol compounds which on oxydation give coloured compounds. Most noticeable is the oxy-

dation of tyrosin to melamin and further to compounds with brown and black colour. Addition of sulphite as sulphurdioxidegas (SO₂-gas) or sulphites gives a reducing environment which prevents the formation of coloured compounds, and at the same time sulphite protects the proteaseinhibitors against damage.

In Norwegian potato starch factories the potato tubers are fineground with high speed grating machines, and the starch granules and the fibers separated from the fruitjuice by a method which is developed in Sweden by the inventor Gösta Larsson. The process makes use of rotating conical sieves, nozzle separators and vacuumfilters. The potato fruitjuice is recirculated and used for transporting the slurry and to wash the starch granules through the fine gauze of the sieves.

Sulphurdioxidegas is added to the potatoslurry to prevent oxydation. Normally about 0.2 kg sulphurdioxide (SO₂ gas)/tonn potatoes is used.

Undiluted potatofruitjuice may also be separated from the finely ground potatomass by the use of decantercentrifuges. Decantercentrifuges are centrifuges with conical, rotating drums where heavy solids move to the periphery and settle to the drum surface and are continuously transported out by means of a screw and leaves the centrifuge. The potatofruitjuice leaves the decantercentrifuge through an adjustable overflow. The solid material is mostly made up of starch and fibers. The starch may be separated from the fibers and worked up into potato starch, or the mixed solid material from the decantercentrifuge may be used as raw material in potatodistilleries.

The potato fruitjuice separated by both these methods may be used in the production of proteaseinhibitor concentrate by the method described in the patentapplication.

By using the invention, the environmental problems previously stated are reduced for the reason that part of the proteins leaves the plant as product, and because one gets a permeate which is well filtered and easy to concentrate by reverse osmosis.

The purpose of the invention is to make use of the potato fruitjuice, which is a waste, as raw material in the production of proteaseinhibitor concentrate. According to the invention this purpose is obtained by the traits given in the characterizing part of claim 1. Additional characterizing traits are given in the dependent claim.

The procedures according to the invention are in detail described in the following.

Heat coagulation of inactive proteins

Proteins for human consumption may be obtained from potatofruitjuice. The proteins are coagulated by making the potatojuice slightly acid and heat it. The coagulated proteins precipitate and the

precipitate is afterwards separated using a decantercentrifuge or in other ways. The same coagulation method is used as pretreatment before concentrating the potatojuice by evaporation or by the use of membraneprocesses, the purpose being afterwards to mix the concentrate with the coagulate removed from the juice and the fibers separated and drying the mix to produce feeding stuffs. By these processes conditions are used where the aim is to obtain a granular proteincoagulate which may be separated from the clear phase using decantersentrifuges or by filtering. Conditions are used which destroy the proteaseinhibitors. There is no information available indicating the use of heat treatment and removal of the coagulate on a technical scale using conditions which retain the biological activities.

The proteaseinhibitors from potato tubers may be divided into more or less heatstable. It has been found and characterized different proteaseinhibitors among the fairly heatstable. This is divided 3 main groups,

- (1) inhibitors with strong chymotrypsininhibitor activity, and
- (2) inhibitors which inhibit strongly both chymotrypsin and trypsin, and
- (3) a polypeptide which inhibit the activities of carboxypeptidase A and B.

The process which is described here, aim at extracting and concentrating the reasonably heatstable inhibitors from the potatojuice.

The result of the coagulation depends on several variables, most important are the following parameters,

- temperature,
- time of heat treatment,
- the pH of the potatojuice,
- concentration of sulphite in the potatojuice.

When the purpose of the treatment is to precipitate as much protein as possible to be separated and used as feed or for consume, hard coagulating conditions are used and a granular coagulate is obtained which is easy to separate from the potato juice. When the aim is to produce a concentrate with good yields of active proteaseinhibitors, it is essential to use mild reaction conditions during the coagulation. Under mild conditions however a coagulate with a soft and slimy structure is obtained which is difficult to separate from the reaction-mixture. We have found that a highspeed plateseparator with nozzle rejection of the concentrate and peridodic sludge emptying and cleaning of the sludgechamber at full speed, gives a clear potato juice which does not block the membranes used for the succeeding concentration by ultrafiltration.

The potatofruitjuice contains about 200 mg sulphite (as SO₂)/litre. Usually sufficient sulphite has been added as sulphurdioxidegas during the starch production. It may however be necessary to adjust the

sulphite concentration during heat coagulation. To add sodium sulphite is a practical way to do this.

The pH of the juice is adjusted by controlled addition of hydrochloric acid. To obtain a good mixing the hydrochloric acid is injected in the pipe line transporting the potato fruit juice to the plant. The addition is automatically controlled by pH measuring and controlled injection of the acid to pH about 4.4.

The heating to coagulation temperature is done continuously in a tube heat exchanger dimensioned for little overtemperatures on the heat-transporting surfaces to avoid local overheating which may destroy inhibitor proteins. Using warm water of 75 - 80 °C as heating medium and a coagulation temperature of 65 - 70 °C measured in the warm juice leaving the heat exchanger after heating for 5 - 10 minutes are preferred conditions. A sufficient precipitation of inactive proteins is obtained this way, and at the same time tolerable loss of inhibitor activity takes place. The conditions depend on potato varieties and the growth conditions used. Formation of coating on the heating surfaces is avoided by processing a not foaming potato juice, and using a low surface temperature of the heat exchanger together with high liquid velocity along the surface. We have found that a liquid velocity of about 1.6 m/sec gives usable conditions.

A high speed disc centrifuge with nozzle outlet of the heavy concentrate together with periodic rejection of sludge and emptying of the sludge-chamber under operation, gives a clear juice does not block the surface of the membranes used for the following concentration by ultrafiltration.

With small production or with production outside the season with starch production, a season which may often be short, a Westfalia decanter centrifuge with a nominal capacity 3.5 m³/hr has been used for extracting the undiluted fruit juice from finely ground potatoes. The solid phase containing fibers and starch and some potato juice has been used as raw material in a nearby distillery. About 60 % of the juice is separated and heated batchwise in a 4 m³ reaction-tank provided with stirrer and an outside heating mantle and a heating coil for circulating water of 80 °C as heating medium. The potato juice was adjusted to pH about 4.4 and 200 mg sulphite (as SO₂)/l added before heating to 65 °C during 20 mins, followed by cooling to 20 °C with cold water through the heat exchanger mantle and coil. The coagulate was removed with a filter press using Kieselguhr (Hyflo) as filter aid.

Concentrating by ultrafiltration

After coagulation and removal of inactive proteins, the clear fluid contains the protease inhibitors. This clarified potato juice is concentrated by ultrafiltration using a relatively low pressure-difference through the membranes. To obtain a good capacity without difficulties, it is important to use a high cross-

flow, i.e. high liquid velocity along the membrane surface, and that the temperature is controlled in such a way that further precipitation of proteins within the membranes does not occur or the membranes are damaged in other ways.

The protease inhibitors are held back in the retentate, as water and small molecules move through the ultrafiltration membranes as permeate. The progress of the concentration step is followed by measurement of the volumes and determination of the inhibitor activities. At present the chymotrypsin inhibitor activity is determined. The juice is concentrated to a predetermined activity. The use of 0.25 l protease inhibitor concentrate to 100 hl fish for fish oil and fish meal production is practical and gives adequate stabilization of the fish raw material. This corresponds to 10 to 15 times concentration of the juice after pre-treatment depending on the quality of the potato raw material.

The concentration is at present done by the use of equipment from DDS-FILTRATION, Denmark, Type 3 x 38 H-5, membranes type ETNA 10 A (polysulfon) and a membrane area of 105 m². The cut-off of the membranes is molecular weight about 10 000 Dalton. Using 4 bar pressure drop through the membranes in the module (5 bar on the inlet side and 1 bar on the permeate side), 30 °C and recirculating about 45 m³/hr over the membranes in the module, the flux on starting up is about 70 l permeate/m²/hr.

Preserving and storing the concentrate

The protease inhibitor concentrate is made non-perishable by the addition of 8% common salt (NaCl), together with the inorganic salts already present in the potato fruit juice, a water activity sufficient to prevent growth of most microorganisms is obtained. The concentrate is stored at low temperature, about 10 °C, and keeps for two years.

Further purification and concentration of the protease inhibitor-concentrate

It is known that proteins, also protease inhibitors, precipitate by the addition of salts. We have found that common salt (NaCl) is suitable for the precipitation of protease inhibitors from potatoes. This is advantageous as the protease inhibitor concentrate already is preserved by the addition of common salt. By adding 150 g salt/l to a concentrate preserved with 80 g salt/l at 10 °C, the protease inhibitors precipitate from solution. The precipitate is removed by centrifugation, washed with a pure solution of salt and filtered under vacuum. The precipitate is dissolved in water and filtered, and salt removed by dialysis or diafiltration. The solution is then concentrated by evaporation under vacuum at 35 °C to a sirup. The remaining water is removed from the sirup by freeze-

rying. A dry product is obtained where 20 % of the protein is proteaseinhibitorprotein.

– the solution is concentrated under vacuum at approximately 35 °C to a sirup, and the remaining water is removed by freeze-drying so that a dry powder is obtained.

Claims

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1. A method for producing proteaseinhibitors CHARACTERIZED THEREBY that as raw material for the process, potatojuice from the production of potato starch is used in such a way that the process includes, 10

- addition of not less than 200 p p m sulphur-dioxide or sulphite to prevent oxydation during the production of potato starch or that sulphur-dioxide or sulphite is added to the potatojuice during the production of the proteaseinhibitors, 15

- adjusting the pH to 4,6 or lower,
- heating to 65 - 70 °C, which is the coagulation temperature of the inactive proteins (globulins) in the potatojuice, with heatexchangers with a controlled surfacetemperature of 75 - 80 °C so that lokal overheating does not damage the proteaseinhibitorproteins, 20 25

- cooling the reactionmixture after the heat-coagulation with a coolingheatexchanger,
- using a highspeed nozzleseparator with a system for throwing away the sludge to get rid of the soft and slimy coagulate, 30

- collection and transport of the clear phase from the nozzle-separator to concentration,
- removal of water by ultrafiltration at about 4 bar pressurefall through the membranes and high cross flow over the membranes untill the predetermined concentration is obtained, the development of the concentration being followed by measurement of volumes and determination of inhibitoractivities, and 35 40

- addition of 7 - 8 % common salt (NaCl) for preserving the proteins and as part of subsequent saltaddition necessary to precipitate active proteins should so be desired.

2. The method according to claim 1, CHARACTERIZED thereby that the procedure also includes production of purified proteaseinhibitors in a dry form by fractional salting out of the proteins by the addition of common salt (NaCl), where the proteaseinhibitors precipitates by the addition of about 150 g salt/litre concentrate with 8 % salt previously added, so that about 70 % saturation is obtained, afterwards 45 50

- removing the sediment by centrifugation or filtration and washing with pure salt solution, 55

- the sediment is dissolved in water, the solution is filtered, and salt removed by dialysis or diafiltration,

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